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Understanding Perturbation in Aquatic Insect Communities under Multiple Stressor Threat

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Abstract

In the scientific literature, there is a considerable consensus that working toward evaluating multiple stressors is worthwhile. Unfortunately, our means to evaluate the combined effects of multiple stressors on species is limited. In agricultural systems, the relative threat posed to aquatic insect communities due to individual stressors (e.g., individual insecticides) is relatively well understood. However, understanding mixtures of pesticides, let alone the addition of complex and potentially interacting, natural gradients (e.g., nutrients and predation), is far harder. The objective of the following review was to evaluate the individual and combined effects of a range of multiple agricultural stressors on aquatic insect communities using a series of seven outdoor mesocosm experiments conducted since 2003. The mesocosm studies show that macroinvertebrate community responses can be similar, subtle, or even opposing depending on the stressors investigated and the mechanistic or ecological focus of the study. The current focus on individual chemicals and responses to treatment is misleading. Cumulative effects and multiple sublethal stressors are the norm in impacted ecosystems. A simple, holistic approach to environmental risk assessment is needed.

Keywords: aquatic communities, multiple stressors, mesocosm experiment, multiple predator theory, insecticides, nutrients, benthic macroinvertebrates, insect predators, review, synthesis

1. Introduction

Streams draining agricultural watersheds contain complex mixtures of pesticides, nutrients, and sediment due to runoff, spray drift, and erosion [1]. Pesticides also tend to be present at sublethal concentration levels at which we even know less about the cumulative toxicity and

multiple stressor threat of mixtures of substances [2]. Some estimates suggest that >50% of river miles in the continental United States include mixtures of five or more pesticides, moderate to highly enriched nutrients and sediments [3]. More recent work has reported similar trends reporting the widespread use of insecticides and neonicotinoids in particular [4–6].

The exposure to mixtures of insecticides and other compounds pose a particular risk to aquatic insects because target biochemical receptors in insects are highly conserved [7]. For instance, the nicotinic acetylcholine receptor (nAChR), the primary binding site for neonicotinoid insecticides in insect pests, has been reported in numerous insect orders (e.g., Hemiptera, Blattodea, Homoptera, Orthoptera, and Diptera) [7]. Among the most highly publicized nontarget species affected by neonicotinoid insecticides are bees (*Apis mellifera*) [8]. Similarly, aquatic insects, such as mayflies (Order: Ephemeroptera), are also negatively affected by exposure to neonicotinoid insecticides at levels associated with agricultural runoff [9, 10]. Responses in other orders of aquatic insects, such as insect predators (e.g., Plecoptera and Odonata), are less studied but preliminary data suggest that these compounds likely affect a wide range of taxa. Knowledge gaps in our understanding of key-stone taxa such as predators may have serious implications for risk assessment as density, and trait-mediated responses may have cascading effects on other members of aquatic food webs [11].

In the literature, there is a considerable consensus that working toward evaluating multiple stressors is worthwhile and important [12–14]. However, there has been virtually no uptake in addressing multiple stressors in ecological risk assessment. This may be due to the complex results emanating from mixture studies, which can be challenging to interpret [15]. Mixture studies are also typically retrospective and rarely address likely combinations of substances [16]. More proactive approaches that examine intentional or unintentional overlap in the field application of chemicals are needed.

The objective of the following studies was to evaluate the effect of multiple, interacting, natural, and anthropogenic stressors on aquatic macroinvertebrate communities. Responses primarily focus on the effects of the neonicotinoid insecticide imidacloprid, individually and in combination, with environmentally relevant mixtures of other substances and changing ecological conditions. Seven mesocosm studies were conducted between 2003 and 2010. Tests included exposure (individually and in mixture) to the following compounds: imidacloprid, the fungicide chlorothalonil, and the organophosphorus insecticides chlorpyrifos and dimethoate. Natural gradients were also examined and included changes in nutrient gradients such as low, medium, and high nutrient enrichments (oligotrophic, mesotrophic, and eutrophic) and increased predation pressure (added stonefly and dragonfly nymphs). Unique to this work is the comparison between responses of aquatic communities tested over time to overlapping treatments all collected from the same riverine source (see Materials & Methods). Further, concentrations selected were within the range of concentrations of pesticides and nutrients that have been detected in runoff and offer new insights as to why some streams become degraded. These findings have never before been summarized; thus, collectively, the following represents a unique snapshot of the range of effects of multiple agricultural stressors on aquatic insect communities.

2. Materials and methods

2.1. Study species

Benthic insects live on the bottom of streams and interact with multiple environmental compartments including water, sediment, and gravel interfaces [17]. Benthic macroinvertebrates (BMI) are good indicators of stream health because changes in BMI diversity and abundance can be associated with some contaminants [18]. Aquatic insects, like midges (Order: Diptera) and mayflies (Order: Ephemeroptera), lend themselves to studies of nutrients and contaminants since they both share many life history characteristics and yet are sufficiently different to highlight changes in streams. Midges in our streams were dominated by the family Chironomidae. Chironomids are small-bodied (adults: 1.5–20 mm [19]) with a short life cycle and emerge throughout the spring, summer, and fall in Atlantic Canada (unpub. data). Like many mayflies, chironomids are often members of the collector-gatherer or scraper trophic guilds, feeding on benthic algae, bacteria, and organic matter. Mayflies are larger than chironomids and may take prolonged periods to develop with some mayfly families only able to emerge once a year [20]. Mayflies are also generally considered to be sensitive to stress, in contrast to the more tolerant midges, and can be good indicators of contamination.

Aquatic insect predators such as dragonflies and stoneflies have also been shown to be sensitive to changes in habitat condition and agricultural gradients, particularly, nutrients [21]. As aquatic nymphs, dragonflies and stoneflies are highly opportunistic predators and show strong allometry to the average body size of their prey [22]. *Gomphus borealis* (Odonata and Gomphidae) are ambush predators that burrow in sediment to await the arrival of suitable prey items [23]. These generalized predators [24] feed by ejecting their labium to grasp their prey before devouring them. In contrast, *Agnetina capitata* (Plecoptera and Perlidae) are foraging predators [25] and search mechanically for prey.

2.2. Study site and allocation of treatments

Since 2003, mesocosm experiments have been conducted at the Environment and Climate Change Canada mesocosm test facility located at Agriculture and Agri-Food Canada, 10-km southeast of Fredericton (New Brunswick, Canada). Among these experiments were a series of studies conducted to examine the effects of multiple stressors on aquatic macroinvertebrate communities. These studies were designed to test the additive, cumulative, and interactive effects of the insecticide imidacloprid, in mixtures of similar (e.g., three insecticides) and dissimilar (insecticide and fungicide) chemicals on aquatic insect assemblages. Test conditions manipulated concentrations of insecticides (imidacloprid, dimethoate, and chlorpyrifos), fungicides (chlorothalonil), nutrients (oligo-, meso-, and eutrophic gradients) and predation pressure (stoneflies and dragonflies). In brief, the chemicals tested were chlorpyrifos (O,O-Diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) and dimethoate (O,O-Dimethyl S-[2-(methylamino)-2-oxoethyl] phosphorodithioate) both organophosphorus insecticides that are among the top 10 most commonly used in North America as well as being highly toxic to nontarget aquatic species [26, 27]. Imidacloprid (1-((6-Chloro-3-pyridinyl)methyl)-N-nitro-2-imidazolidinimine) is a neonicotinoid insecticide,

while chlorothalonil (2,4,5,6-tetrachloro-1,3-benzenedicarbonitrile) is a widely used fungicide in Atlantic Canada [28, 29].

The experiments were designed to evaluate a range of conditions (**Table 1**) for example, (1) a chronic, low nutrient (oligotrophic) study conducted in the Fall of 2003 (22 September 2003–21 October 2003) that explored continuous exposure to the insecticide imidacloprid in the lethal effects range; (2) a pulse, low nutrient (oligo-mesotrophic boundary) study conducted in the Summer of 2004 (20 June 2004–10 July 2004), which combined a chronic and a pulse experiment that explored lower concentrations of the same range of insecticide exposures with the addition of some nutrients (e.g., [TN] $25 \pm 3 \mu\text{g/L}$) described in [10]; (3) a pulse, mesotrophic nutrient enrichment study conducted in the Fall of 2004 (3 August 2004–1 September 2004) that included the addition of moderate nutrients (as above and [TN] $30 \pm 4 \mu\text{g/L}$); (4) a pulse, low nutrient study conducted in the Fall of 2005 (4 August 2005–24 August 2005) and an imidacloprid-chlorothalonil mixture experiment that explored the same range of insecticide exposures and nutrients see [30]; (5) a binary (1:1) mixture of two insecticides chlorpyrifos and dimethoate (12 July–2 August 2007) [31]; (6) a ternary (1:1:1) mixture of three insecticides chlorpyrifos, dimethoate, and imidacloprid (16 August–6 September 2009) [21]; and (7) a pulsed imidacloprid within a nutrient gradient study conducted in 2010 (17 July–6 August 2010) see [32].

For each study, 80 artificial streams or outdoor mesocosms (**Figure 1**) were inoculated with a benthic macroinvertebrate community collected in the Nashwaak River, New Brunswick,

Experiment	Exposure duration in -d or -h	Stressors tested (ppb)	References
1. Chronic (press), oligotrophic study	20-d	Imidacloprid (5, 15)	
2. Press vs. pulse, oligo-mesotrophic study	20-d or 12-h	Imidacloprid press (0.1, 0.5, 1) and pulse (0.1, 0.5, 1, 5, 10)	[10]
3. Sublethal (pulse), mesotrophic study	24-h (2×) or 24-h (4×)	Imidacloprid (0.5, 1)	
4. Pesticide mixture (pulse), oligo-mesotrophic study	24-h (3×)	Imidacloprid (0.6, 17.6) Chlorothalonil (3, 30)	[30]
5. Insecticide mixture (pulse), oligo-mesotrophic study	96-h (1×)	Chlorpyrifos (1, 2, 4) Dimethoate (5, 10, 20)	[31]
6. Insecticide mixture (pulse), oligo-, and mesotrophic study	96-h (1×)	Imidacloprid (0.5, 1, 2) Chlorpyrifos (0.5, 1, 2) Dimethoate (2, 4, 8)	[21]
7. Nutrient-insecticide (pulse), oligo-, meso- and eutrophic study	96-h (1×)	Imidacloprid (1.4, 5)	[32]

All experiments were conducted over a 20-d period. Concentrations of stressors tested given in parts per billion (ppb), throughout.

Table 1. Overview of the design of seven mesocosm experiments conducted between 2003 and 2010.

Canada (46°8'34.584" N × 66°22'1.992" W). Each flow-through stream was circular and had a planar area of 0.065 m² and a 10-L volume. Each treatment level contained at least eight replicate streams. Treatment levels varied depending on the test objective but are summarized in detail elsewhere (see **Table 1**). Throughout, chemical analyses determined the actual concentrations of pesticides (National Laboratory for Environmental Testing, ECCC Saskatoon) and nutrients (RPC Fredericton). In brief, pesticide analyses were conducted on a Micromass Quattro Ultima liquid chromatography mass spectrometer (LC-MS/MS) with Waters 2695 Alliance HPLC System equipped with a Waters Xterra MS C18 (100 × 2.1 mm i.d., 3.5 µm particle size, Milford, MA, USA) analytical column. Samples were routinely collected on multiple occasions during and after the exposure period. Pesticide samples were stored in 500 ml amber vials (EPA vials, Fisher scientific, Fair Lawn, NJ, USA) and stored at 4°C until shipment to Saskatoon for analysis. Nutrient treatments were chosen based on Biggs [33] and corroborated using in-stream chlorophyll-*a* measurements compared to levels reported in Dodds et al. [34]. Water quality samples and emergent insects were collected daily throughout each experiment.

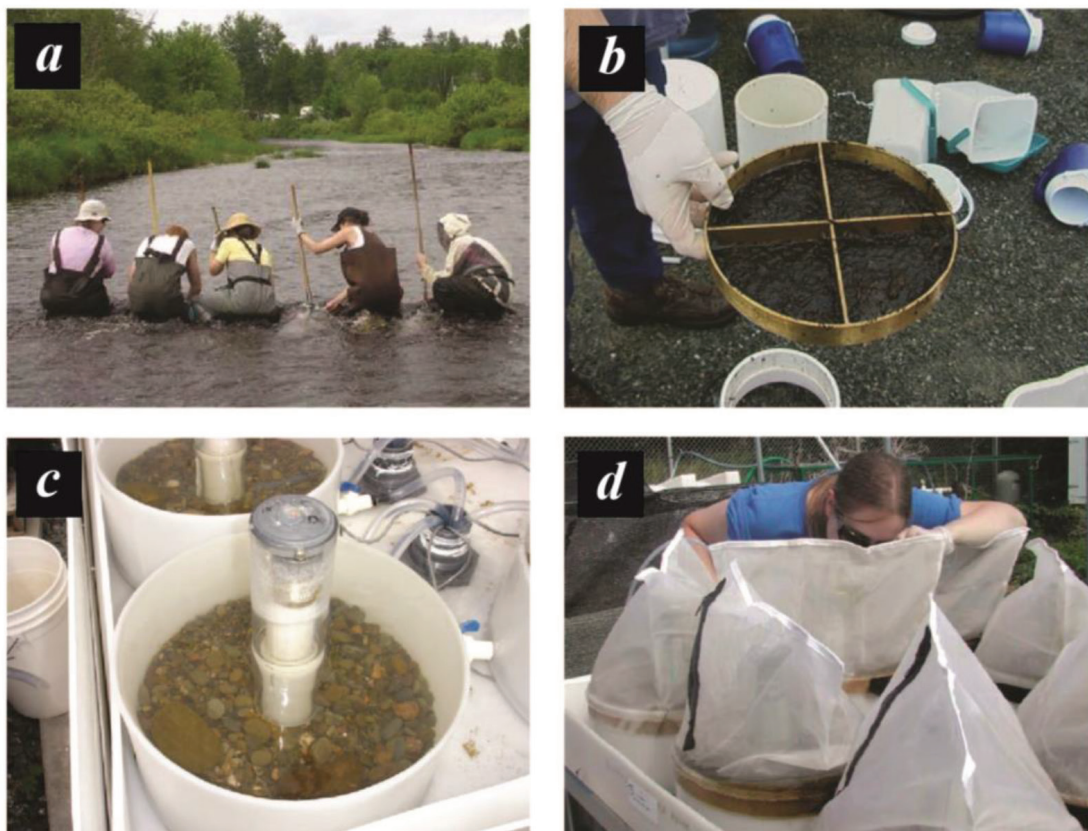


Figure 1. Outdoor, flow-through, stream mesocosms. (a) Benthic macroinvertebrates are collected by five samplers collecting 4 U-nets each. (b) The benthic community is then subsampled (four-way pie-plate subsampler shown). Community subsamples are then inoculated into replicate streams (e.g., ¼ of community sampled per replicate). (c) Each replicate stream is circular (0.065 m² and 10-L volume) and was also inoculated with five cobblestones and coarse and fine gravel. (d) After inoculation with benthic macroinvertebrates each stream is covered with 45 µm mesh to facilitate the daily collection of emergent insects.

2.3. Ecological endpoints

At the end of each 20-d mesocosm experiment, the streams were dismantled and the contents collected. Water samples, periphyton samples, and invertebrates were collected from each replicate stream. For chlorophyll-*a* ($\mu\text{g}/\text{cm}^2$) and ash-free dry mass (AFDM, mg/cm^2), three scrapings (each 60.2 cm^2) were collected into 20-mL scintillation vials and frozen in a portable freezer at -20°C (Engel fridge/freezer MT35F-U1, Sawafugi Electric Co. Ltd., Tokyo, Japan). Aquatic nymphs and emergent adults were then measured using the Auto-Montage imaging program (Syncroscopy, Synoptics Inc., Frederick, MD, USA) with a Leica digital camera and dissecting microscope (Leica Microsystems Ltd., Cambridge, UK). Multiple photographs were taken of each organism and measurements were conducted on segments using linear and curvilinear measurement tools. Calibrations were conducted for each objective lens and were repeated for individual insect measurements if the coarse or fine focus was adjusted. Numerous measurements were taken, including maximum head length and width, maximum thorax length and width, wing pad length, and total body length. In the absence of wing pads, the total length of the thorax was measured from the center of the anterior tip of the pronotum dorsally to furthest posterior point along the centerline of the metanotum. When wing pads were present, the total length of the thorax was measured from the center of the anterior tip of the pronotum dorsally to furthest posterior tip of the wing pad along the left lateral axis. Predation pressure was estimated as the product of the density (per cm^2) and body size (mm) of predators such as the stonefly *Agneta capitata* and dragonfly *Gomphus borealis* per replicate stream (described in [32]).

2.4. Statistical analysis

Responses were examined using a complement of standard parametric (ANOVA) and multivariate statistical tools including: (e.g., nonmetric multidimensional scaling, factor analysis, principal components analysis as well as mixed general linear and structural equation models) see [35–37]. Assumptions of statistical tests were met throughout. Differences in river subsamples and control tanks were assessed using the Euclidean distance method to compare the distance of reference samples calculated by the unweighted pair group method [38]. Structural equation models (SEM) were used to assess changes in food webs between treatment levels and were estimated using covariance in partial regression coefficients [39]. Finally, principal components analysis was used to confirm the strength of relationships due to nutrient treatment.

3. Results

Seven mesocosm experiments were conducted between 2003 and 2010 (**Table 1**). Responses varied between studies but the pesticide or nutrient treatment applied were major drivers of changing patterns in the macroinvertebrate community. Changes over time due to successional or seasonal changes in the sampled aquatic community were less evident than those due to pesticide or nutrient treatment. For instance, at the onset of the mesocosm experiments, subsampled river communities were similar to other subsamples collected during the same period (**Figure 2**). River communities were also similar to assemblages observed in control streams at the end of the 20-d mesocosm experiment (**Figure 2a**). However, treatment with neonicotinoid insecticides such as imidacloprid (5 or 15 ppb, 20-d press exposure) resulted in

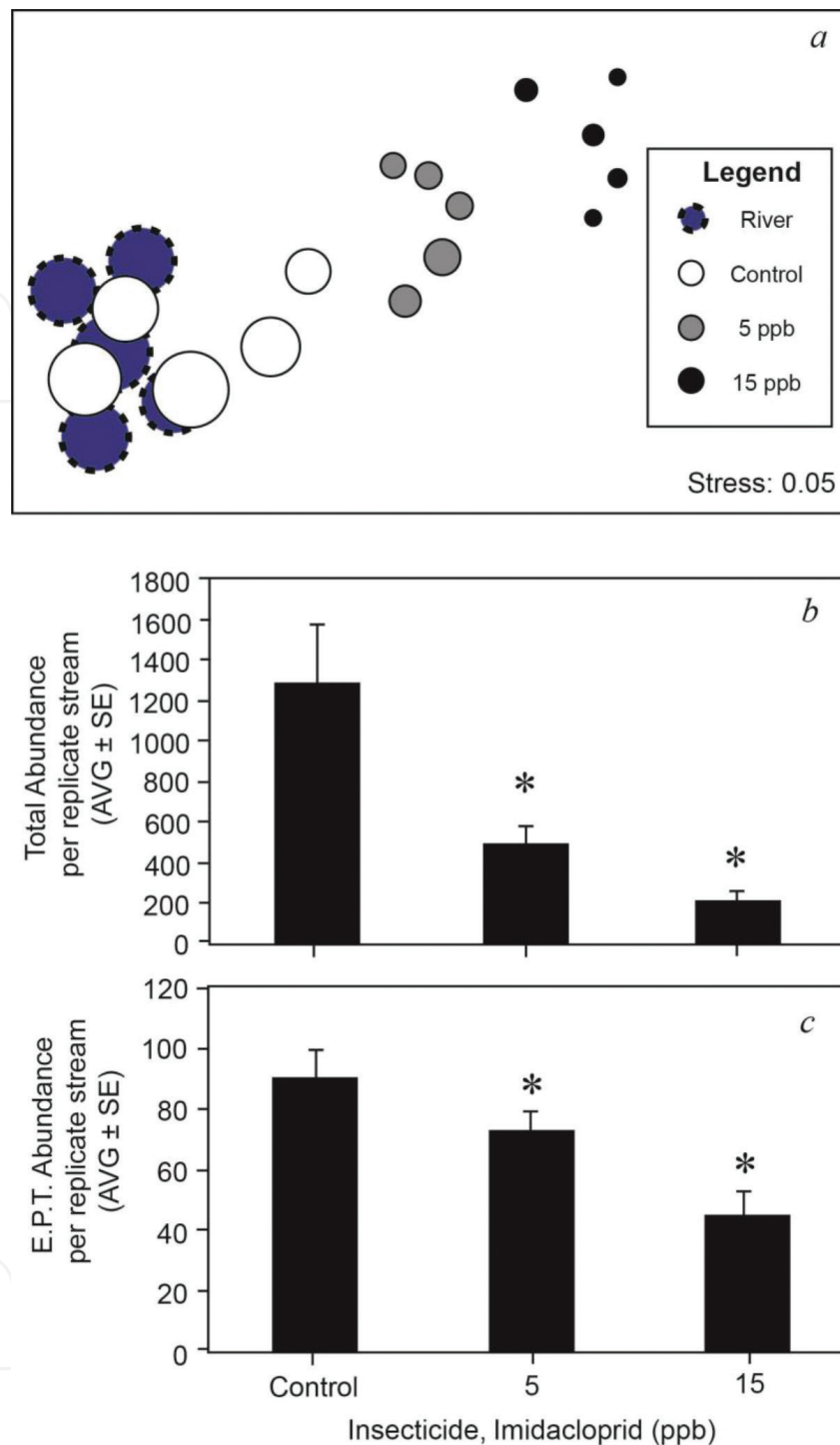


Figure 2. Benthic macroinvertebrate community responses (a) nonmetric multidimensional scaling (nMDS) to treatment with three concentrations (control, 5 and 15 ppb) of the neonicotinoid insecticide, imidacloprid, in the 2003 pilot mesocosm study. The size of the circles reflects the abundance of organisms and the distance between circles the magnitude of change between replicate communities. (b) Total abundance of aquatic macroinvertebrates per replicate stream (AVG total no. per stream \pm SE). (c) Abundance of sensitive E.P.T. (orders Ephemeroptera, Plecoptera, and Trichoptera) aquatic insect taxa per replicate stream (AVG total of E.P.T. only per stream \pm SE). Significant differences ($P < 0.05$) are indicated (*).

major changes in the abundance and diversity of aquatic insect taxa (**Figure 2a**). For example, severe reductions (>78 and 92% in 5 and 15 ppb) in the total abundance of taxa (**Figure 2b**) and sensitive E.P.T. taxa were strongly associated with imidacloprid treatment (>18 and 49%;

see **Figure 2c**) (e.g., $F_{3,30} \geq 5.43$, $P < 0.01$). Further, experiments examining an increasing range of imidacloprid concentrations demonstrated similar and significant decreases in community total abundance, total richness, and E.P.T. abundance (e.g., Mesocosm #1, $F_{2,14} \geq 5.90$, $P \leq 0.01$; Mesocosm #4: $F_{2,71} \geq 3.30$, $P \leq 0.05$) (**Table 1**).

Nutrient treatment also differed between studies (**Table 1**). Enrichment could be measured as changes in periphyton abundance (as chlorophyll-*a* in $\mu\text{g}/\text{cm}^2$) and was consistent with the nutrient treatment applied (low to high enrichment: oligo-, meso-, or eutrophic). Responses to nutrient enrichment were consistent irrespective of the year of study or seasonal changes in the macroinvertebrate community. Community responses to the combined action of nutrients and insecticides could also appear similar. For instance, the removal of insect grazers (structural change) at the base of the food web in high insecticide treatments was associated with increased periphyton biomass (functional change). Thus, oligotrophic streams treated with imidacloprid were more similar to mesotrophic or even eutrophic conditions due to grazer release despite the lack of nutrient enrichment (e.g., $3.3 \pm 0.5 \mu\text{g}/\text{cm}^2$ due to 15 ppb treatment with imidacloprid) ($F_{2,23} = 3.91$; $P = 0.03$).

A factor analysis of benthic macroinvertebrate community responses to treatment explained 45% of the variance in all of the community data collected between 2003 and 2009 (Cumulative Eigenvalue 21.38) (**Figure 3**). Throughout, responses to treatment differed ($P < 0.05$) between Factor 1 (E.V. 17.68 of 21.38, 37%) and Factor 2 (E.V. 7.73 of 21.38, 45%). Factor 2 was closely correlated with the magnitude (concentration \times duration) of imidacloprid concentration ($r = 0.65$, $P < 0.05$) and Factor 1 reflected differences associated with community composition (e.g., presence, absence, and diversity). In control streams, macroinvertebrate community responses to oligotrophic and mesotrophic enrichment overlapped, whereas responses to eutrophic treatment were discernibly separated from those in lower levels of nutrient enrichment (**Figure 3a**). Treatment with a single insecticide also overlapped for similar chemical compounds such as the insecticides imidacloprid, dimethoate, and chlorpyrifos ($P > 0.05$) (**Figure 3b**). In contrast, community responses to dissimilar chemicals, such as mixtures of imidacloprid and nutrients, diverged from those of imidacloprid alone (**Figure 3c**). Community responses also diverged in response to the combined action of imidacloprid, nutrients, and increased predation pressure (**Figure 3c**). Interestingly, community responses to mixtures of imidacloprid and the fungicide chlorothalonil were similar despite differences in the mode of action of these two compounds (**Figure 3c**).

A structural equation model of the covariant relationships between different organisms, trophic guilds, and other metrics (e.g., periphyton biomass) was also used to compare food webs in the nutrient enriched (mesotrophic) versus limited (oligotrophic) streams (**Figure 4**). In oligotrophic streams, only two response variables significantly covaried ($P < 0.05$) (**Figure 4a**). Specifically, the density (no./ cm^2) of the dragonfly *Gomphus borealis* covaried with ash-free dry mass, or AFDM (mg/cm^2), but did not covary with the density of other predators, such as the stonefly *Agnetina capitata* or the abundance of scrapers (**Figure 4a**). Rather, the density of *A. capitata*, covaried with scrapers ($P < 0.05$), which in turn may be associated with chlorophyll-*a*, but only at the $P < 0.1$ level. In contrast, mesotrophic streams had 17 covariant relationships ($P < 0.05$) between different taxa and guilds (**Figure 4b**).

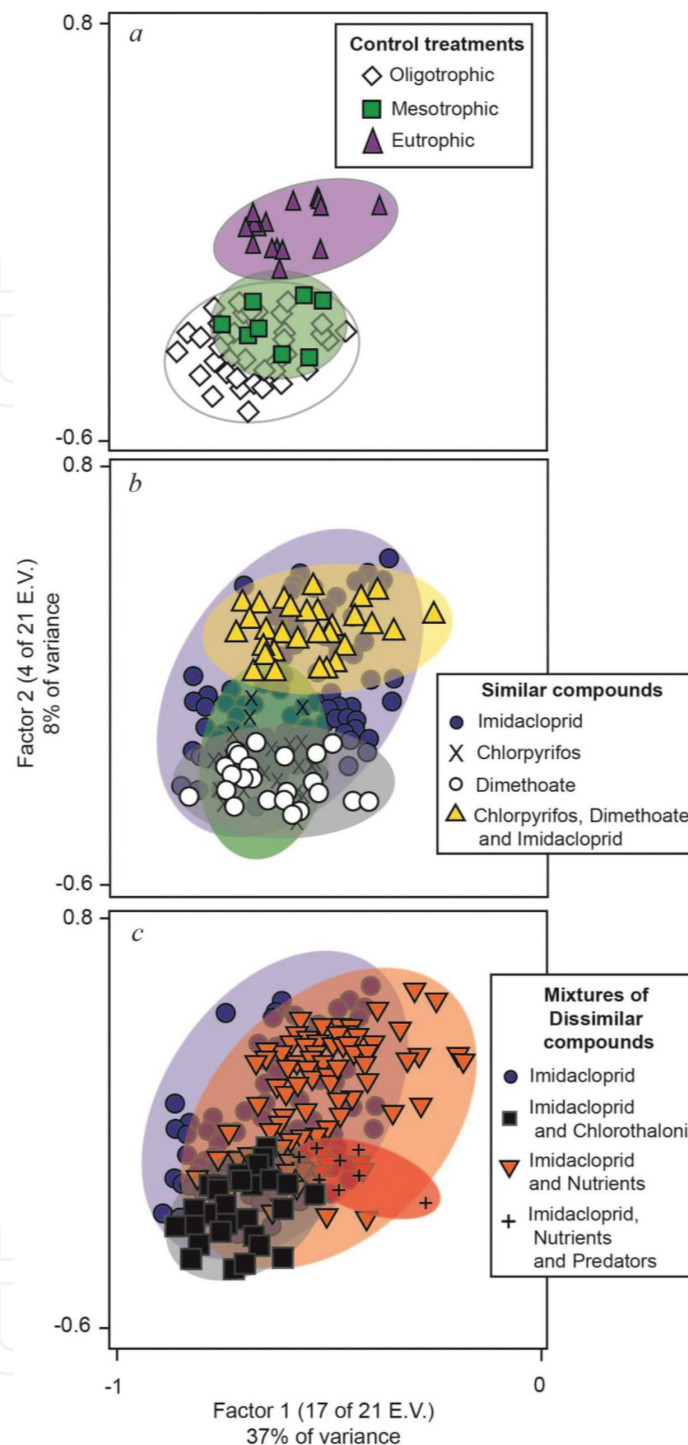


Figure 3. Factor analysis of benthic macroinvertebrate community abundance (no. of different genera per treatment level) during 7 years of mesocosm experiments subdivided into (a) control treatments with the addition of no nutrients (oligotrophic), moderate nutrients (mesotrophic), and high nutrients (eutrophic). (b) Exposure to similar insecticides either individually (imidacloprid, chlorpyrifos, and dimethoate) or in mixture (all three insecticides), and (c) exposure to mixtures of dissimilar chemical contaminants (as mixtures only). Dissimilar contaminants tested included the insecticide imidacloprid, and fungicide chlorothalonil, imidacloprid in the presence of nutrient enrichment (mesotrophic or eutrophic) and imidacloprid in the presence of mesotrophic nutrients and stonefly predators. Ellipses enclose all replicate treatment responses at the 95% CI. Lack of overlap between ellipses suggests statistically significant differences between responses to treatment at the $P < 0.05$ level.

For instance, the density of *G. borealis*, covaried ($P < 0.05$) with the density of its main competitor, *A. capitata*, as well as with other predators. Collectively, *G. borealis* and *A. capitata* both covaried with the density of a range of taxa including consumers from multiple sensitive orders (E.P.T. consumers), as well as scrapers, collector-gatherers, and shredders (**Figure 4b**). In turn, these taxa, and collector-gatherers in particular, affected the density of other guilds (e.g., E.P.T. consumers, collector-filterers, and piercers) as well as the standing stock of the periphyton community (AFDM, chlorophyll-*a*) (**Figure 4b**). Eutrophic conditions were only examined in a single mesocosm study (#7, conducted in 2010, see **Table 1**), and as such, relationships between taxa and guilds are less generalizable than those reported for oligotrophic and mesotrophic streams.

Responses, however, within eutrophic streams overlapped those in oligotrophic and mesotrophic nutrient treatments as well as with specific stressor conditions unique to Mesocosm #7, the only eutrophic gradient tested (**Figure 5** and **Table 1**). Genera and guilds

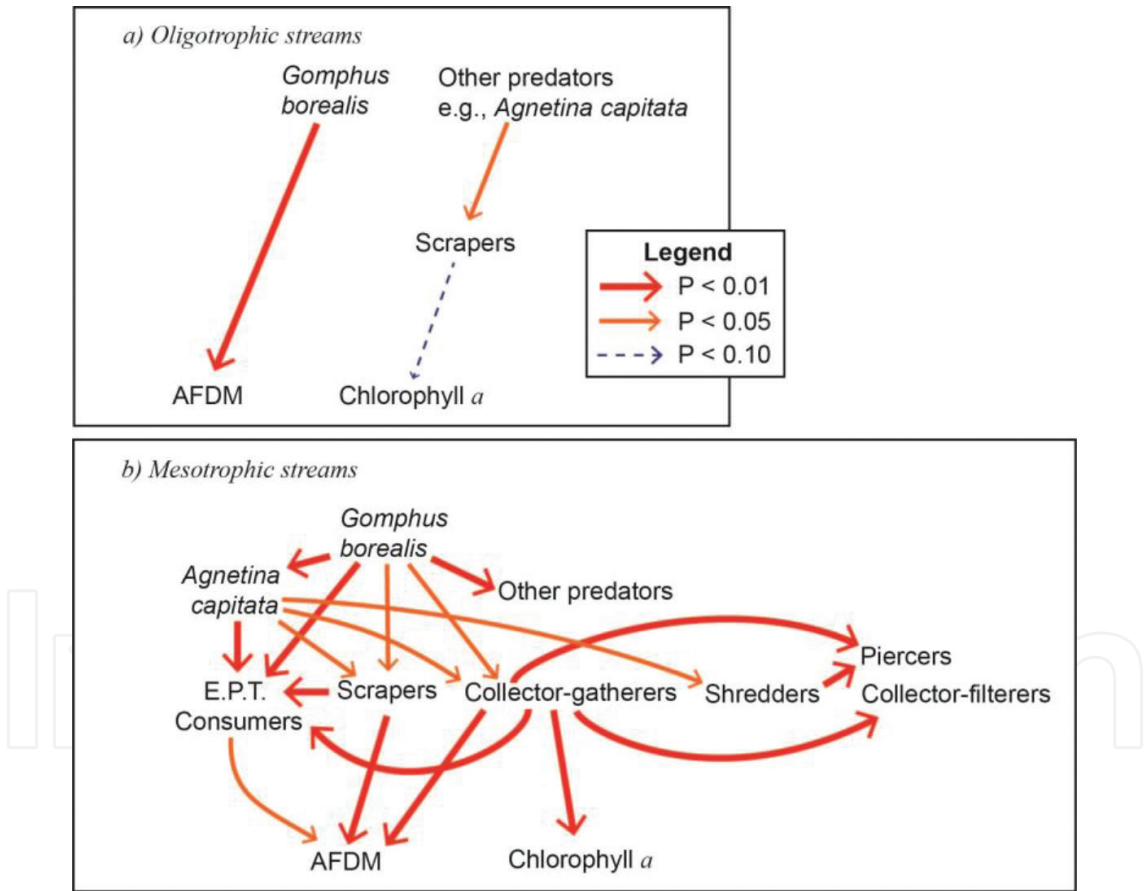


Figure 4. Summary of significant covariant relationships between the density (no./cm²) of different taxa, guilds and other metrics in control streams under oligotrophic (a) or mesotrophic (b) nutrient treatment. (a) Only two significant covariant relationships were reported under nutrient limited (oligotrophic) conditions whereas under (b) moderately nutrient enriched conditions (mesotrophic), 17 covariant relationships between taxa, guilds, or periphyton biomass were evident (measured as chlorophyll *a* in µg/cm² (chlorophyll) were found).

tended to respond similarly to treatment, and 68% of the variance in macroinvertebrate density could be explained by treatment with nutrients or the insecticide imidacloprid (52.3% of Factor 1 and 15.9% of Factor 2, **Figure 5**). For instance, total abundance, E.P.T. abundance, total richness, and density of collector-gatherers were all primarily ($r \geq 0.72$, Factor 1) responding to the combined action of nutrient and insecticide gradients and secondarily to nutrient treatment specifically ($r \leq 0.63$, Factor 2). In contrast, chlorophyll *a* and AFDM were only highly correlated ($r = -0.68$ and $r = -0.71$) to Factor 2. Finally, communities in control eutrophic streams were most similar to oligotrophic streams that were simultaneously treated with concentrations that are lethal to 50% of the insect population (median lethal concentration or LC50). Thus, in eutrophic streams, concentrations that would be highly significant stressors in less enriched streams were closely related to responses associated with baseline condition in these highly enriched systems (**Figure 5**).

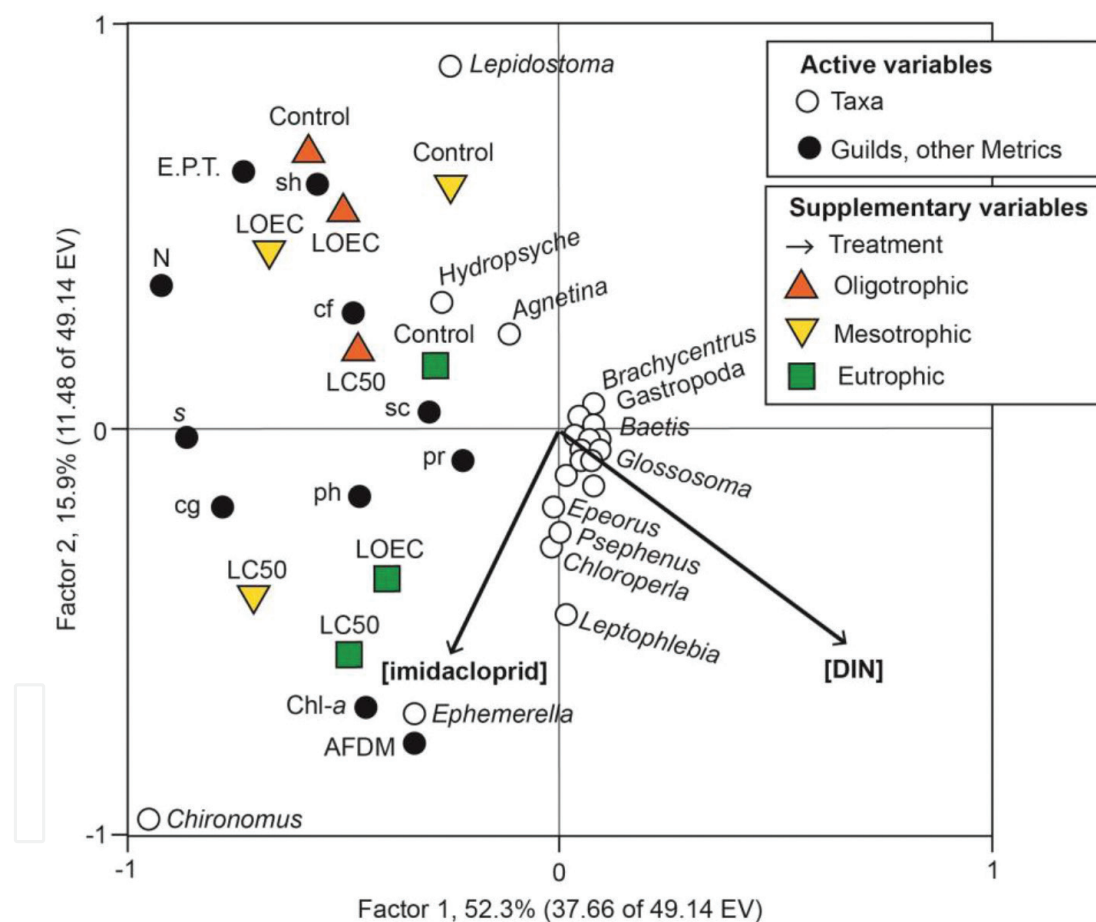


Figure 5. Principal components analysis of mesocosm 7 only (17 July to 6 August 2010) explaining 68% (52.3 + 15.9% EV) of the variation in benthic macroinvertebrate community (no./stream/cm²) and periphyton biomass (chl-*a* in µg/cm² and AFDM mg/cm²) due to either nutrient enrichment (oligotrophic, mesotrophic or eutrophic due to the addition of dissolved inorganic nitrogen [DIN]) or neonicotinoid insecticide treatment (imidacloprid as control, lowest observable effect concentration [LOEC], or median lethal concentration [LC50]). Density of select genera and guilds are highlighted; for example, total abundance (N), richness (s), E.P.T. abundance (E.P.T.), collector-filterers (cf), collector-gatherers (cg), piercers (ph), predators (pr), scrapers (sc) and shredders (sh). All comparisons were made using a correlation matrix.

4. Discussion

Streams draining agricultural catchments contain complex and often sublethal mixtures of pesticides and nutrients [1]. Ecological risk assessments rarely consider chemical mixtures, let alone combinations of natural and anthropogenic gradients. Regulators focus on individual compounds. Pesticides are regulated in Canada using a risk ranking approach based on an evaluation of the presence of available application data (e.g., sales or max application rate), chemical fate information (e.g., persistence and mobility), and toxicity (e.g., single species toxicity tests on fish, invertebrates, or aquatic plants). This focus on mortal responses to individual compounds poses a problem because it fails to consider conditions that are common in the environment: sublethal mixtures of chemicals are widespread. It is also evident that single species laboratory tests of individual compounds cannot approximate mixtures of chemicals affecting interacting assemblages of organisms in ecosystems.

The results of the studies described above show that in combination, pesticides and nutrients can reshape food webs (see also [9, 10, 21, 30–32]). In isolation, the action of these stressors appears to supersede underlying seasonal differences in macroinvertebrate communities. This finding suggests that nutrients and pesticides are fundamental drivers of effects in impacted aquatic communities. However, macroinvertebrate responses to pesticides and nutrients were varied and responses may be structurally similar yet functionally different. In the studies described above, responses due to nutrients and insecticides, such as the neonicotinoid and imidacloprid, were difficult to discern. The removal of grazers (**Figure 2**) at the base of the food web also increased periphyton biomass to levels that would suggest moderate or even high levels of enrichment ($> 3 \mu\text{g}/\text{cm}^2$) despite the lack of added nutrients (Mesocosm #1, in 2003). Further support for this finding is found in a separate experiment (Mesocosm #7, in 2010) where eutrophic streams were structurally and functionally similar to nutrient-limited streams simultaneously dosed with lethal doses (LC50) of imidacloprid (**Figure 5**). Collectively, these findings suggest that cascading effects at one end of the food web are common but could be due to different, and potentially, interacting pathways.

At lower doses, community responses to stress tended to overlap (**Figure 3b**) [10, 21]. For instance, communities were structurally similar due to low dose mixtures of three insecticides (chlorpyrifos, dimethoate, and imidacloprid) or due to any of these same compounds when tested individually at moderate or even high doses (**Figure 3b**). However, differences in community structure could be subtle as responses to treatment with mixtures of different types of compounds (e.g., pesticides vs. nutrients) tended to have less overlap when co-exposed to either substance individually (**Figure 3c**). Further evidence for structural changes in aquatic communities due to nutrients is apparent in the structural equation model (**Figure 4**). The covariant relationships between taxa varied widely between nutrient enriched versus limited streams despite the same aquatic macroinvertebrate assemblage being initially introduced into each treatment level.

Varied responses to different types of chemical compounds may appear to make ecological risk assessment difficult (see Kienzler et al. [16] for a review of approaches). Currently, in Canada, risk rankings list the toxicity of chemical compounds to different types of taxa (invertebrates,

fish, or plants) using data collected from single species toxicity tests. For instance, fish toxicity ranks include different pesticides than rankings developed for invertebrates or plants. Specifically, the top three pesticides that are thought to pose the greatest risk to invertebrates are the neonicotinoid insecticides imidacloprid, thiamethoxam, and clothianidin. These same neonicotinoids are ranked as being far lesser risk of toxicity to fish (9, 20, and >30) or plants, respectively (>30). At present whether these substances are likely to co-occur is not considered.

There are advantages to the joint testing of substances. For instance, by testing effects jointly the number of tests to be conducted may decrease as only relevant mixtures need testing. Joint testing will also deepen our understanding of dose-dependent effects of similar and dissimilar mixtures of chemical compounds offering new insights into the likelihood of synergistic and antagonistic effects. The advantage of increased environmental realism is also of critical importance and will aid in the development of better monitoring programs and regulations. Computer simulations, for instance, based on the chemical mode of action (e.g., Quantitative Structure Activity Relationships (QSARs) see [40]) are an important first step to reduce the time and cost of more detailed assessments while promoting informed decision making.

Joint exposure to multiple stressors has been addressed previously in the ecological literature in the theory of multiple predators (e.g., [11, 41–43]). The multiple predator approach is particularly fitting, as responses to predators are highly variable (e.g., [44]) as are responses to insecticides (e.g., above studies). The theory of multiple predators shows that predator-predator interactions can cause conflicting risk to prey and lays out a framework for assessing the emergent properties of multiple predators on simple food webs. In the ecological framework, each predator is treated as an individual stressor and as such presents an interesting analogy to work with different chemical stressors. The predator framework modified for chemical stressors suggests that there are a series of steps to move forward with cumulative effects risk assessment. These are: (1) to define the criteria for identifying mixtures of likely substances, (2) monitor how common substances interact with each other and environmental compartments, (3) assess what mechanisms may underlie unexpected interactions, and (4) propose how the impacts of multiple stressors on stream communities may be regulated. This approach is far simpler than some of the chemical-based approaches suggested by others while also enabling the inclusion of insights gained using these methods [45, 46]. Finally, a simple, holistic approach that integrates ecological components will likely present a fresh perspective enabling the capture of the complexity of both the mixtures of chemicals under investigation and the interacting assemblages of organisms in real ecosystems.

5. Conclusions

Complex mixtures of five or more pesticides, as well as nutrients and sediments, are pervasive in the aquatic environment. Yet, mortal endpoints of single chemicals on single species laboratory tests are the norm in regulatory frameworks. A more holistic approach is needed. Within the regulatory community, there is a concern that multiple stressor studies are difficult to interpret and as a result, are often ignored. The above synthesis and review

of seven mesocosm studies on the combined effects of pesticides, nutrients, and macroinvertebrate community dynamics show that interactions between chemical substances, nutrient enrichment, and trophic status can change how communities respond to stress. This work offers unique insights into the evaluation of multiple stressors as it shows that expected toxic mechanisms can be muted or intensified in response to changing natural and anthropogenic gradients. This finding of diverse responses to stress is consistent with findings from field studies in the literature where some communities tend to be more resilient to stress than others. Understanding multiple stressor effects in an ecological framework (e.g., theory of multiple predators) within a regulatory context may offer a simple and more holistic approach to environmental risk assessment integrating findings from mixture theory and community-level responses to multiple stressors.

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Conflict of interest

The author declares no conflict of interest.

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